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(54) Title: USE OF A MILK PROTEIN HYDROLYSATE IN THE TREATMENT OF DIABETES

(57) Abstract: Use of a milk protein hydrolysate which is preferably a whey protein hydrolysate or caseinoglycomacropeptide (CGMP) in a bioavailable form in the manufacture of a composition for the treatment or prevention of diabetes or syndrome X and a method of treatment or prevention of diabetes or syndrome X are described. The present invention also relates to a method for assessing proglucagon gene expression and GLP-1 release by a cell line derived from an adenocarcinoma of human caecum.

Use of a Milk Protein Hydrolysate in the Treatment of Diabetes

FIELD OF THE INVENTION

The present invention relates to the use of milk protein hydrolysates in the manufacture of a medicament for the treatment or prevention of diabetes or syndrome X and a method of treatment of diabetes or syndrome X which comprises administering an effective amount of a composition comprising milk protein hydrolysates. The present invention also relates to the use of sweet whey or acid whey proteins or protein hydrolysate in the manufacture of a medicament for the treatment or prevention of diabetes or syndrome X and a method of treatment of diabetes or syndrome X which comprises administering an effective amount of a composition comprising sweet or acid whey proteins or protein hydrolysate. Furthermore, the present invention also relates to the use of CGMP in the manufacture of a medicament for the treatment or prevention of diabetes or syndrome X and a method of treatment of diabetes or syndrome X which comprises administering an effective amount of a composition comprising CGMP.

In addition, the present invention relates to the use of NCI-H716 cells, obtained from a cell line derived from a poorly differentiated adenocarcinoma of human caecum (de Bruine et al, Virchows Archiv B Cell Pathol 62:311-320,(1992)), as a model to measure proglucagon gene expression and GLP-1 secretion.

BACKGROUND OF THE INVENTION

B. Chabance et al. (Biochimie 80, 155-165, 1998) have shown that after eating, many peptides derived from α-, β- or κ-caseins, including CGMP, can be detected in stomach and blood and this indicates that it can cross the intestinal barrier.

Diabetes mellitus is a metabolic disorder characterised by the failure of body tissues to store carbohydrates at the normal rate. Resistance to the action of insulin is the most important factor to type II diabetes. When this resistance exceeds the capacity of the beta cells to produce insulin, a person becomes diabetic. During the last 70 years people suffering from diabetes have been greatly aided by receiving controlled amounts of insulin.

Historically, insulin has been administered by injection to combat diabetes. Administering an injection requires expertise, and compared to oral administration, injecting a medicament is not as safe, convenient or acceptable to the patient. In the light of these concerns it is clear that there is a need for new nutritional or therapeutic products which may be administered orally.

Proglucagon, synthesised by L-cells found in the distal ileum and colon, is known to be post-translationally processed into peptides including glucagon-like peptide-1 (GLP-1), a potent insulin secretagogue. In addition to potentiating glucose-induced insulin secretion, GLP-1 is known to stimulate proinsulin gene expression and proinsulin biosynthesis.

Other actions of GLP-1 include inhibition of glucagon secretion and gastric motility. GLP-1 can bind in the brain, promoting satiety and suppressing food intake. Increasing insulin secretion is a key goal in the treatment of type II diabetes and stimulation of endogenous release of GLP-1 is a potential/prospective alternative to intravenous administration.

Improving glucose control in diabetes can provide the advantage of reducing the associated risks of hyperglycaemia, including blindness, limb amputations, kidney failure and cardiovascular disorders.

A number of *in vitro* cell models of animal origin have been developed to study the regulation of GLP-1 secretion including a foetal rat intestinal cell culture, a isolated canine L cell, a secretin tumour cell (STC-1) cell line, and the GLUTag enteroendocrine cell line. While these models have provided useful information regarding the factors which regulate GLP-1 secretion and proglucagon expression, they suffer from the problem that they do not necessarily represent the same regulators and mechanisms which are active and occur in human L cells.

The present invention addresses the problems set out above.

SUMMARY OF THE INVENTION

Remarkably, it has now been found that a milk protein hydrolysate can induce the release of GLP-1 and it can be used to improve glucose homeostasis *in vivo*.

In fact, in contrast to known studies, NCI-H716 cells have now been employed, obtained from a cell line derived from a poorly differentiated adenocarcinoma of human caecum (de Bruine et al, Virchows Archiv B Cell Pathol 62:311-320,(1992)). Surprisingly, the NCI-H716 cell line has now is found to be a good model for the first study of potential secretagogues, which regulate human GLP-1 secretion, *in vitro*. Up to date, NCI-H716 cell line was not known to be suitable for this purpose. Nor is it known from any human cell line to be capable of releasing GLP-1. So far, only cell lines derived from animals were available to serve as *in vitro* models to study proglucagon gene expression and GLP-1 secretion. This property of said cell line will enable much simplified research on GLP-1 release. Furthermore, the results obtained by the use of a human cell line to conduct studies on the production or function of GLP-1 will be much more relevant than result derived from other animal models. In short, the NCI-H716 cell-line line derived from a poorly differentiated adenocarcinoma of human caecum is likely to become a key tool for studying proglucagon gene expression and GLP-1 secretion in human. This cell line is deposited and available at the ATCC (American Type Culture Collection) under the ATCC Number CCL-250. The Depositor is A. F. Gazdar and the tissue of origin is the caecum, it is derived from a colorectal adenocarcinoma.

Consequently, in a first aspect the present invention provides use of a milk protein hydrolysate which is capable of inducing release of GLP-1 in the manufacture of a composition for the treatment or prevention of diabetes or syndrome X.

In a second aspect the invention provides a method of treatment or prevention of diabetes or syndrome X which comprises administering an effective amount of a milk protein hydrolysate which is capable of inducing release of GLP-1.

In a third aspect, the present invention provides a model for the study of proglucagon gene expression and GLP-1 production by humans comprising cells obtained from a cell line derived from an adenocarcinoma of human caecum.

In further aspect, the present invention provides a method for assessing proglucagon gene expression and GLP-1 release in humans comprising a cell line derived from an adenocarcinoma of human caecum.

In a last aspect, the present invention teaches the use of a cell line derived from an adenocarcinoma of human caecum to assess proglucagon gene expression and GLP-1 release in vitro.

An advantage of the present invention is that it provides a composition, which can be administered orally. This is both safer and more convenient for the patient than conventional treatment by injection.

Another advantage of the present invention is the reduced risk of hypoglycaemic reactions. Conventional injection of insulin carries with it the undesirable side effect of hypoglycaemic reactions. The use of oral hypoglycaemic agents to augment insulin secretion can also result in hypoglycaemic reactions. Once the plasma glucose levels reach fasting values, GLP-1 no longer stimulates insulin release. The advantage of enhancing insulin release via GLP-1 secretion is that the action of GLP-1 is glucose dependent and therefore eliminates the risk of hypoglycaemia, i.e. the release of insulin is very fine-tuned with respect to the blood glucose levels actually present.

Yet another advantage is that GLP-1 remains active in persons with diabetes whereas the other incretin hormone, glucose dependent insulinotropic peptide (GIP) loses effectiveness in diabetes.

Still another advantage of the present invention is that it provides metabolic benefits in addition to the augmentation of insulin release. Conventional treatment raises insulin levels, but the present invention in addition increases insulin mRNA, increases beta-cell sensitivity, and lowers glucagon levels.

Another advantage of the present invention is that it provides a composition, which can regulate appetite and reduce food intake. This action is beneficial in control of diabetes and syndrome X.
Still another advantage of the present invention is that for the first time a human cell line for the study of proglucagon gene expression and GLP-1 release is used.

Additional features and advantages of the present invention are described in, and will be apparent from, the description of the presently preferred embodiments, which are set out below with reference to the drawings in which:

DESCRIPTION OF THE DRAWINGS

Figure 1 shows secretion of GLP-1 by NCI-H716 cells in response to incubation for 2 h with different doses of CGMP-Ca form. Secretion into the medium was normalised to the total GLP-1 content (secretion + cells) of the culture well and is expressed as a percentage of the control value.

Figure 2 shows secretion of GLP-1 by NCI-H716 cells in response to incubation for 2 h with different doses of CGMP-Na form. Secretion into the medium was normalised to the total cell content of the culture well and is expressed as a percentage of the control value.

Figure 3 shows secretion of GLP-1 by NCI-H716 cells in response to incubation for 2 h with different fractions of CGMP. Secretion into the medium was normalised to the total cell content of the culture well and is expressed as a percentage of the control value. The composition of the different fractions was the following:

Fraction 1) Hydrolysed CGMP, pure peptide material, no phosphorus, no sialic acid.
Fraction 2) Hydrolysed CGMP, high sialic acid, high phosphorus content. Sample is in the Na-form.
Fraction 3) CGMP fraction enriched in CMPa and CMPb, the phosphorylated compounds of CGMP. Sample is in the Na-form.
Fraction 4) CGMP fraction enriched in sialic acid. Sample is in the Ca-form.

Figure 4 shows the amount of GLP-1 released in the medium after a 2h incubation period in the presence of 5 mg/ml of sweet whey, acid whey and meat protein hydrolysates. To exclude a possible effect due to the alpha-lactose content of the fractions of whey protein hydrolysates, an equivalent alpha-lactose dose as the one contained in the different wheys is separately illustrated.

In Figure 4, The GLP-1 secreted was measured differently than in Figures 1 to 3, i.e. with a kit that only measures the active form of GLP-1, i.e. GLP-1(7-37) or GLP-1(7-36 amide), but not the degraded GLP-1(9-36 amide) form like in figures 1 to 3 (see methods).

DETAILED DESCRIPTION OF THE INVENTION

Within the context of this specification the word "comprises" is taken to mean "includes, among other things". It is not intended to be construed as "consists of only".

Within the context of this specification the term "milk protein hydrolysate" is taken to mean milk proteins that have been subjected to any sort of hydrolysis. Thus, such "milk protein hydrolysate" may even include intact proteins that escaped hydrolysis and also any sort of fractions of proteins as obtained by the treatment of hydrolysis.

Within the context of this specification the terms "sweet whey" and "acid whey" are also considered to be possible milk protein hydrolysates, because they are the product of enzymatic or acid hydrolysis of milk proteins. Whey, however, as is well known in the art, can also comprise intact protein as well as different fractions of hydrolysed protein.

CGMP is used as an abbreviation for caseino-glycomacropeptide and CGMP-Ca and CGMP-Na are used as abbreviations for the calcium salt and sodium salt thereof. An alternative name for caseino-glycomacropeptide is k-caseinoglyco-peptide.

CGMP is a milk protein hydrolysate. It is a sialylated macropeptide, which is formed by the action of rennet or pepsin on kappa-casein from the milk of mammals.

Preferably, the milk protein hydrolysate which is capable of inducing release of GLP-1 comprises CGMP, a mimetic, homologue or fragment thereof which retains the activity of CGMP.

Preferably, an embodiment of the milk protein hydrolysate comprises the calcium or sodium salt of CGMP.

Preferably, the composition comprises a source of carbohydrate, a source of fat and a source of protein. More preferably, it comprises from about 15 to about 25% protein, from about 10 to about 30% fat, and from about 40 to about 60% carbohydrate. Preferably, at least a portion of the protein is provided as protein from sweet whey or acid whey. More preferably, at least a portion of the protein is provided as caseinoglycomacropeptide (CGMP).

Preferably, the milk protein hydrolysate, which is capable of inducing release of GLP-1, comprises proteins that are present in sweet whey or acid whey.

Preferably the composition is incorporated into a food formula.

Preferably, the composition comprises from about 1 to about 50 grams, preferably from 5 to about 25 grams and most preferably from 5 to about 10 grams protein hydrolysate from sweet whey or acid whey, or CGMP alone, or acid whey without CGMP, or a mixture thereof per 100 g of food formula.

Preferably the composition is administered to provide sufficient whey protein or, CGMP alone or whey protein without CGMP, to improve glucose metabolism in humans or companion animals by increasing plasma GLP-1 levels and controlling glycemic response. The exact amount could be determined without difficulty by administering whey protein or, as an example, CGMP until the correct effect is seen. The dose of whey protein comprising or not comprising CGMP or of CGMP itself is preferably from about 1 to about 50 grams per day,

more preferably from 9 to about 18 grams per day and most preferably from 3 to about 6 grams consumed at three times throughout a day.

It is well known in the art how a milk or whey protein can be obtained. In general, skimmed milk is treated with enzymes or acid in order to finally separate sweet or acid whey, respectively, which is thus deprived from the clotted casein. The sweet or acid whey then comprises whey protein hydrolysates and also minor proteins, which remain intact. Thus, sweet or acid whey is, for example, obtained as a side product from production of cheese. Although it is not necessary to additionally process whey to work the present invention, it is obvious to the skilled person that further processing is possible. For example, sweet and acid fluid whey can be condensed, dried, fermented, delactosed, demineralized and deproteinated. In order to work the present invention it is, for example, possible to use whey concentrate or whey powder. The latter is especially convenient to be added to any chosen food product to cause the desired effect. It is also clear to the skilled person, that protein hydrolysate present in sweet or acid whey can be further hydrolysed, for example to prepare a hypoallergenic whey protein hydrolysate. According to U.S. Pat. No. 5,039,532, whey protein material is subjected to a second hydrolysis with a proteolytic enzyme in order to hydrolyse the minor proteins remaining intact after the first hydrolysis. Such a hydrolysate may then be used as a liquid or it may be dried and incorporated in numerous food products.

Preferably CGMP is obtained by an ion-exchange treatment of a liquid lactic raw material containing CGMP. Suitable starting materials of lactic origin may include for example:

- the product of the hydrolysis with rennet of a native casein obtained by acidic precipitation of skimmed milk with a mineral acid or acidifying ferments, optionally with addition of calcium ions,
- the hydrolysis product of a caseinate with rennet,
- a sweet whey obtained after separation of casein coagulated with rennet,
- a sweet whey or such a whey demineralised, for example, by electrodialysis and/or ion exchange and/or reverse osmosis,
- a concentrate of sweet whey,
- a concentrate of whey proteins obtained by ultrafiltration and diafiltration of sweet whey.

- mother liquors of the crystallisation of lactose from a sweet whey,
- a permeate of ultrafiltration of a sweet whey.

A preferable method of obtaining CGMP is described, for example, in WO 98/53702 and includes the decationization of the liquid raw material, such that the pH has a value of 1 to 4.5, bringing the said liquid into contact with a weak anionic resin of hydrophobic matrix, predominantly in alkaline form up to a stabilised pH, then separation of the resin and the liquid product which is recovered, and desorption of CGMP from the resin.

Preferably, an embodiment of the composition comprises a milk protein hydrolysate. It has been shown that skimmed milk results in CGP levels of 1.1μg/ml in human plasma. After yoghurt ingestion 2.8μg/ml of CGP has been detected in blood.

Preferably, an embodiment of the composition comprises CGMP.

Preferably, an embodiment of the composition comprises sweet or acid whey, more preferably, intact or partially hydrolysed proteins from sweet or acid whey.

Preferably an embodiment of the composition comprises a source of protein and at least protein hydrolysates from sweet whey or acid whey, or CGMP. Dietary protein is preferably used as a source of protein. The dietary proteins may be any suitable dietary protein; for example animal protein (such as milk protein, meat protein or egg protein); vegetable protein (such as soy protein, wheat protein, rice protein, or pea protein); a mixture of free amino acids; or a combination thereof. Milk protein such as casein, whey protein or soy protein is particularly preferred.

The composition may also contain a source of carbohydrate and/or a source of fat.

A preferred embodiment of the composition comprises a fat source, the fat source preferably provides about 5% to about 55% of the energy of the nutritional formula; for example about 20% to about 50% of the energy. The lipids making up the fat source may be any suitable fat or fat mixture. Vegetable fat is particularly suitable; for example soy oil, palm oil, coconut oil, safflower oil,

sunflower oil, corn oil, canola oil, lecithins, or the like or a mixture thereof. Animal fat such as milk fat may also be added if desired.

A preferred embodiment of the composition comprises a source of carbohydrate. It preferably provides about 40% to about 80% of the energy of the nutritional composition. Any suitable carbohydrate may be used, for example sucrose, lactose, glucose, fructose, corn syrup solids, and maltodextrins, or a mixture thereof.

A preferred embodiment of the composition comprises dietary fibre. If used, it preferably comprises up to about 5% of the energy of the nutritional formula. The dietary fibre may be from any suitable origin, including for example soy, pea, oat, pectin, guar gum, gum arabic, or fructooligosaccharide.

A preferred embodiment of the composition comprises one or more suitable vitamins and/or minerals may be included in an embodiment of the composition in an amount to meet the appropriate guidelines.

A preferred embodiment of the composition comprises one or more food grade emulsifiers may be incorporated into the nutritional formula if desired; for example diacetyl tartaric acid esters of mono- and di- glycerides, lecithin and mono- and di-glycerides. Similarly suitable salts and stabilisers may be included.

A preferred embodiment of the composition is enterally administrable; for example in the form of a powder, a liquid concentrate, or a ready-to-drink beverage. If it is desired to produce a powdered nutritional formula, the homogenised mixture is transferred to a suitable drying apparatus such as a spray drier or freeze drier and converted to powder.

In a further embodiment, a typical food product may be enriched with whey protein or CGMP. For example, a fermented milk, a yoghurt, a fresh cheese, a renneted milk, a confectionery bar, breakfast cereal flakes or bars, drinks, milk powders, soy-based products, non-milk fermented products or nutritional supplements for clinical nutrition. Then, the amount of whey protein or of CGMP added is preferably of at least about 0.01% by weight.

In an alternative embodiment the composition may be incorporated in an article of confectionery, for example a sweet, or sweetened beverage.

MATERIALS AND METHODS

Materials for the cell culture:

RPMI 1640 medium, Dulbecco's Modified Eagles medium (DMEM), additives and foetal bovine serum (FBS) were from Gibco (Life Technologies, Basel, Switzerland). Bovine serum albumin (BSA) was purchased from Serological Proteins Inc. (Kankakee, IL).

Materials for testing the effect of CGMP:

CGMP was obtained from R&D Konolfingen and was dissolved directly in Krebs Ringer Buffer. Two forms of CGMP, sodium extracted and calcium extracted, were tested as well as four fractions of CGMP.

Materials for testing the effect of sweet and acid whey:

For this study, conventional milk fractions at lab scale from fresh bovine milk (local market) were prepared.
Rennet (Presure simple) was from Rhône Poulenc Rorer (Cooperation Pharmaceutique Française, 77000 Melun France, Batch N°101089007 expire date 2000.09.21) 50 mg active chymosine by liter. Produced by TEXEL 38470 Vinay France. Furthermore, CaCl2 2H2O, HCl 32%, Acetic acid (glacial), Sodium Hydroxide were used.

Milk fractions

Bovine milk fractions

Lab scale fractionments were adapted from conventional milk processes. Centrifugation was realised at higher acceleration rate and non-soluble fractions were washed to increase selectivity and fractionment efficacy.

Cream; cream washing and skimmed milk

Usually cream was extracted from whole milk by centrifugation between 3 000 and 4 500 g, the selectivity of this step was improved by increasing acceleration up to 13 600 g using fixed angle rotor Sorval GS3 at 9 000 rpm during 30 min. Starting from 2 200 ml of whole milk 90g of cream were recovered on the top layer.

Cream washing (3 times labelled respectively, *cream washing1, 2 and 3*): the cream layer was dispersed in 3 water volumes (270 ml) and gently scattered in bottle by manual shaking before subsequent centrifugation.

Butter particles were spontaneously formed at the top of the bottle after the third cream washing and the buttermilk was recovered by sticking butter particles together.

A non-soluble fraction was recovered after centrifugation on the bottom of bottles used for cream washings (Labelled: *washed cream sediments*)

Sweet whey, rennet casein washing and rennet casein

The separation whey/casein is obtained by enzymatic treatment of skimmed milk inducing casein clotting. 520µl of CaCl2 200mM were added to 520g of skimmed milk to reach 2 mM final concentration added. This skimmed milk was heated at 35 °C then 250µl of rennet were immediately added under moderate magnetic stirring. After 1 min the blend was incubated 50 min at 35°C in water bath, poured in bottles for subsequent centrifugation (13 600g 30 min) to separate sweet whey from the non-soluble rennet casein.

The 476 g of supernatant were fractionated in 10 X 1.3 ml aliquots (ependorf) and 40 ml plastic tubes. Labelled (*Sweet whey*) and freezed by immersion in liquid nitrogen and stocked in plastic bag at minus 20°C.

The rennet casein (45g) was dispersed in 286 ml CaCl2 2mM NaCl 0.9%, centrifuged, the supernatant (246 ml) was aliquoted, labelled (*rennet casein washing*) and freezed in liquid nitrogen.

The 31g recovered rennet casein were dispersed in CaCl2 2mM NaCl 0.9%, volume was adjusted to 250 ml, aliquoted, labelled (*rennet casein*) and freezed.

Acid whey, acid casein washing and acid casein

The separation whey/casein is obtained by chemical acidification of skimmed milk inducing casein clotting. 520µl of CaCl2 200mM were added to 520g of skimmed milk to reach 2 mM final concentration added. This skimmed milk was acidified at 25°C by addition of 32% HCl from pH 6.6 to pH 4.6 °C under moderate magnetic stirring. After 1 min of stirring the blend was incubated 60 min at 25°C, poured in bottles for subsequent centrifugation (13 600g 30 min) to separate acid whey from the non soluble acid casein.

The 503 g of supernatant were fractionated in 10 X 1.3 ml aliquots (ependorf) and 40 ml plastic tubes. Labelled (*Acid whey*) and freezed by immersion in liquid nitrogen and stocked in plastic bag at minus 20°C.

The acid casein (41g) was dispersed in 233 ml 20mM Sodium acetate pH 4.6, centrifuged, the supernatant (250 ml) was aliquoted, labelled (*Acid casein washing*) and freezed in liquid nitrogen.

The 28.6g recovered acid casein were dispersed in water, pH adjusted from 4.67 to 6.6 by NaOH addition and volume was adjusted to 250 ml, aliquoted, labelled (*Acid casein*) and freezed.

Cell line and culture conditions:

The human NCI-H716 cells, originally developed from a poorly differentiated caecal adenocarcinoma, were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were grown in suspension at 37°C, 5% CO_2. The culture medium consisted of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Endocrine differentiation is enhanced *in vitro* in NCI-H716 cells grown on an extracellular matrix (de Bruine et al, 1993). Cells were, therefore, seeded in dishes coated with Matrigel® (Becton Dickinson, Bedford, MA, USA) two days before experiments.

Secretion Studies:

Two days before experiments, $1x10^6$ cells were seeded in 12 well culture plates coated with Matrigel®. On the day of the experiment, the supernatant was replaced by Krebs-Ringer Bicarbonate Buffer (KRB) containing 0.2% wt/vol

BSA with or without CGMP. Cells were incubated for 2 h at 37°C. Supernatants were collected with the addition of 50 µg/ml PMSF and frozen at –80°C for subsequent analysis by radioimmunoassay (RIA) of GLP-1. Cells were scraped with a rubber policeman and homogenisation buffer [1 N HCl containing 5% (v/v) HCOOH, 1% (v/v) trifluoroacetic acid (TFA), and 1% (v/v) NaCl] and sonicated for 15s. Peptides were extracted from the cell medium and cell homogenates using an alcohol extraction as described by the supplier of the GLP-1(7-36) Total RIA Kit (Linco Research Inc., St. Charles, MO, USA). Concentrations of GLP-1 (Total, i.e., 7-36 amide or 9-36 amide) were measured using a commercial RIA kit (Linco Research Inc., St. Charles, MO, USA). This kit measures GLP-1(7-36)NH_2 and GLP-1(9-36)NH2 with less than 0.4% crossreactivity with GLP-1(7-37). The ED_{50} for the assay was 72 pM. The intraassay coefficient of variance was 2.28%.

For testing the effect of whey and meat hydrolysates, (figure 4), the GLP-1 secreted was measured differently than before, i.e. with a commercial ELISA kit (Linco Research Inc., St. Charles, MO, USA). This kit measures the active form of GLP-1, i.e. GLP-1(7-37) or GLP-1(7-36 amide), but not the degraded GLP-1(9-36 amide) form like in figures 1 to 3.

RESULTS

CGMP stimulates the release of GLP-1 in the NCI-H716 intestinal cell line.

The amount of GLP-1 released into the medium of NCI-H716 cells treated for 2h with increasing concentrations (0.25-2.5 mg/ml wt/vol) of the calcium form of CGMP is shown in Fig. 1. CGMP induced a dose-dependent increase in GLP-1 concentrations with maximum secretion reaching 259 ± 77% (n = minimum of 3) of the control values with 2.5 mg/ml of CGMP-Ca. The symbol * represents a significant difference from control values ($p<0.05$).

Figure 2 shows the amount of GLP-1 released into the medium of NCI-H716 cells treated for 2h with increasing concentrations (0.25-2.5 mg/ml wt/vol) of the sodium form of CGMP. CGMP induced an increase in GLP-1 concentrations with maximum secretion reaching 255 ± 41% (n = minimum of 3) of the control

values with 2.5 mg/ml of CGMP-Ca. The symbol * represents a significant difference from control values ($p<0.05$).

Figure 3 shows the amount of GLP-1 released into the medium of NCI-H716 cells treated for 2h with 1 mg/ml (wt/vol) of different fractions of CGMP. All fractions, except fraction 3 ($p=0.085$) significantly increased GLP-1 secretion with Fraction 2 resulting in the highest stimulation of 220 ± 41% ($n=3$) of the control values. The symbol * represents a significant difference from control values ($p<0.05$).

Sweet and acid whey stimulate the release of active GLP-1 in the NCI-H716 intestinal cell line.

The amount of GLP-1 released in the medium after a 2h incubation period in the presence of 5 mg/ml milk protein hydrolysates is shown in figure 4. Sweet whey and acid whey induced an increase in GLP-1 release of 298 ± 34 % and a 284 ± 21 %, respectively, compared to control condition ($p = 0.03$ and 0.01 respectively, $n = 3$). This effect was not due to the alpha-lactose content of these fractions, as an equivalent alpha-lactose dose as the one contained in the different wheys only resulted in a small raise in GLP-1 secretion (144 ± 32 %, compared to control). Moreover, another protein hydrolysate, meat hydrolysate, didn't induce such an effect on GLP-1 production, at 5 mg/ml (132 ± 6 %, compared to control).

The following examples are given by way of illustration only and in no way should be construed as limiting the subject matter of the present application. Percentages and parts are by weight unless otherwise indicated.

Example 1: Preparation of CGMP.

Bovine sweet whey was concentrated to 17% dry matter, demineralised by electrodialysis, freed of cations on a strong cationic resin column, freed of anions on a weak anionic resin column and spray-dried in a drying tower. Its composition is indicated below:

	%
Proteins (GMP included)	11.7
Lactose	81.7
Ash	1
Lipids	1
Water	balance for 100

The demineralized whey powder was solubilized in deionized water. After cation removal the solution has an initial pH of 3.8. In the preceding plant, 392 kg of this solution was treated at the temperature of 8°C, while stirring it in the reactor in the presence of 23 kg of weak anionic resin of hydrophobic matrix based on polystyrene (IMAC HP 661®, Rohm & Haas, regenerated in OH- form) for 4 h. Stabilization of the pH at 4.89 indicates the end of the reaction. The liquid was drawn off and the resin was recovered as above.

After concentration of the liquid to 45% dry matter by evaporation, the concentrate was spray-dried in a drying tower.

Analysis of the concentrate by HPLC showed that the reaction removed 89% of the starting CGMP. Moreover, the powder contained 9.1% of whey protein, which corresponded to a yield of 90% of the whey proteins.

To recover CGMP, the resin was washed successively with deionized water, with 30 l of an aqueous solution at 0.5% HCl and with 30 l of deionized water, and the CGMP was eluted twice with 40 l of aqueous solution at 2% Ca(OH)2. Rinsing is carried out with 30 l of deionized water. After combining the eluate and rinsing volumes, the combination was concentrated to a volume of 25 l by ultrafiltration with a membrane having a nominal cut-off of 3000 daltons. The retentate was freeze-dried and 900 g of CGMP were obtained, corresponding to a yield of 80% relative to the starting CGMP.

Example 2 : Fermented milk containing CGMP or whey powder

A traditional fermented milk with 1-4 % fats was prepared as follows:
After standardising whole milk, low fat milk or a mixture of both, 0.05% by weight of CGMP as prepared in example 1 are added. The whole was pasteurised

in a plate exchanger, the liquid was cooled to the fermentation temperature, a thermophilic or mesophilic lactic ferment was added and incubation was carried out until a pH of <5 was obtained.

Subsequent filling and sealing pots took place in a conventional manner.

Alternative embodiments having additions of 0.1 %, 0.25 % and 0.5% by weight of CGMPs and commercial whey powder have been prepared.

Example 3 : Fermented and gelled milk enriched in probiotic bacteria containing CGMP or whey powder

Fermented and gelled milks were prepared enriched in probiotic bacteria. 89.3 parts milk containing fat were mixed with 3.7 parts of skimmed milk powder and about 0.05 by weight of CGMP as prepared in example 1, then the mixture was preheated to 70°C and pasteurised at 92°C/6 min, and after having been cooled to 43°C the mixture was inoculated with 2% of a common yoghurt starter comprising Streptococcus thermophilus and Lactobacillus bulgaricus and with 5% of Lactobacillus johnsonii (La-1, CNCM I-1225). After conditioning in pots, fermentation was carried out at 38°C up to pH 4.6 and the pots were then cooled to 6°C.

The following amounts of CGMP or commercial whey powder were added: 0.1 %, 0.25 % and 0.5% by weight.

Example 4 : Fermented and gelled milk enriched in probiotic bacteria containing CGMP or whey powder

Fermented and gelled milks are prepared as described in the previous example, wherein Lactobacillus johnsonii strain is replaced by Lactobacillus acidophilus La-10 (Nestlé Culture collection, Lausanne, Switzerland) (ATCC 11975).

Example 5 : Enteral composition containing CGMP

An enteral composition with an energy density of 6.3 kJ/ml and 8% (p/v) of proteins was prepared from "low temperature" skimmed milk powder, i.e. skimmed milk dried under controlled thermal conditions.

20 kg of low temperature skimmed milk powder was dispersed in 100 kg of demineralised water at a temperature of about 50-55°C. This dispersion is microfiltered by passing demineralised water through until 600 kg of permeate have been eliminated. The retentate is then further concentrated to around 60 kg, which represents a dry matter content of 21% with a protein content, based on dry matter, of 82%.

To prepare the enteral composition, 2.3 kg of liquid retentate are mixed at 55°C with 600 g of maltodextrin, 200 g of sucrose, 20.3 g of Tri-K citrate H_2O, 9.2 g of $MgCl_26H_2O$, 5.8 g of NaCl and about 0.5 to 1 % by weight of CGMP as prepared in example 1 or, instead of CGMP, commercial whey powder.

After the ingredients were dissolved in the retentate, demineralised water is added to a total weight of the dispersion of 4.7 kg. The pH was adjusted to 6.8, after which 300 g of fatty phase are introduced, the total weight of the dispersion being 5 kg.

After homogenisation and sterilisation, the product had an agreeable sugary taste.

Example 6 : Cereal bar containing CGMP or whey powder

In order to prepare an expanded starting product, barley, wheat, corn or oat flour was treated in a twin-screw extruder for about 15 seconds at a screw speed of about 350 r.p.m. in the presence of approximately 12% of water. After the treatment, the expanded product left the extruder in the form of 2 to 3 mm long granules which were dried for 20 minutes at 100°C. The product thus obtained had a cellular structure and has the following composition:

Edible fibers	31%
Proteins	21%
Glucides	37.5%
Lipids	6.5%
Ash	2.4%
Water	1.6%

The expanded product was incorporated in a bar intended for treatment of diabetes, which had the following composition:

Expanded product	39.4%
Oat flakes	16.7%
Sorbitol	8.4%
Fructose	8.5%
Apple cubes	6.1%
Rice crispies	4.1%
Gelatine	4.0%
Apricot powder	2.5%
Palm oil	3.0%
CGMP as prepared in example 1	2.5%
Water	4.8%

Example 7: Food supplement containing CGMP

A culture of the strain Lactobacillus johnsonii La-1 (CNCM I-1225) of human origin, was mixed with CGMP as prepared in example 1 and spray dried according to the process given in EP0818529 so as to obtain a food supplement containing an amount of about 5% by weight of CGMP.

The powder obtained may be used as a food supplement. A breakfast cereal, milk product or another food product may then be sprinkled with this powder containing CGMP.

Example 8: Food supplement containing CGMP

A food supplement was prepared as described in example 9. However, Lactobacillus johnsonii was replaced by Lactobacillus acidophilus, La-10 (Nestec collection, Lausanne, Switzerland) or a mixture of the two strains.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

CLAIMS

1. Use of a milk protein hydrolysate or compound including a milk protein hydrolysate, which is capable of inducing release of GLP-1, in a bioavailable form in the manufacture of a composition for the treatment or prevention of diabetes or syndrome X.

2. Use according to claim 1 wherein the milk protein hydrolysate is caseinoglycomacropeptide (CGMP), a mimetic, homologue or fragment thereof.

3. Use according to claim 2 wherein CGMP is in the form of a calcium or sodium salt.

4. Use according to any preceding claim wherein the composition comprises an amount of about 0.01% to about 10% by weight dry matter of CGMP.

5. Use according to claim 1 wherein the milk protein hydrolysate is sweet whey or acid whey.

6. Use according to claim 5 wherein the composition comprises an amount of 0.01% to about 10% by weight dry matter of sweet or acid whey.

7. A method of treatment or prevention of diabetes or syndrome X which comprises administering an effective amount of a milk protein hydrolysate which is capable of inducing release of GLP-1.

8. A method according to claim 7 wherein the milk protein hydrolysate which is capable of inducing release of GLP-1 is CGMP, a mimetic, homologue or fragment thereof

9. A method according to claim 8 wherein the CGMP is in the form of a calcium or sodium salt.

10. A method according to claim 7, wherein an effective amount of sweet or acid whey, or further processed sweet or acid whey is administered.

11. A model for the study of proglucagon gene expression and GLP-1 production by humans comprising cells obtained from a cell line derived from an adenocarcinoma of human caecum.

12. A model according to claim 11, characterised in that said cell line is the NCI-H716 cell line, having the ATCC number CCL-251.

13. A method for assessing proglucagon gene expression and GLP-1 release in humans comprising a cell line derived from an adenocarcinoma of human caecum.

14. A method according to claim 13, characterised in that said cell line is the NCI-H716 cell line, having the ATCC number CCL-251.

15. The use of a cell line derived from an adenocarcinoma of human caecum to assess proglucagon gene expression and GLP-1 release in vitro.

16. The use according to claim 15, characterised in that said cell line is the NCI-H716 cell line, having the ATCC number CCL-251.

Figure 1

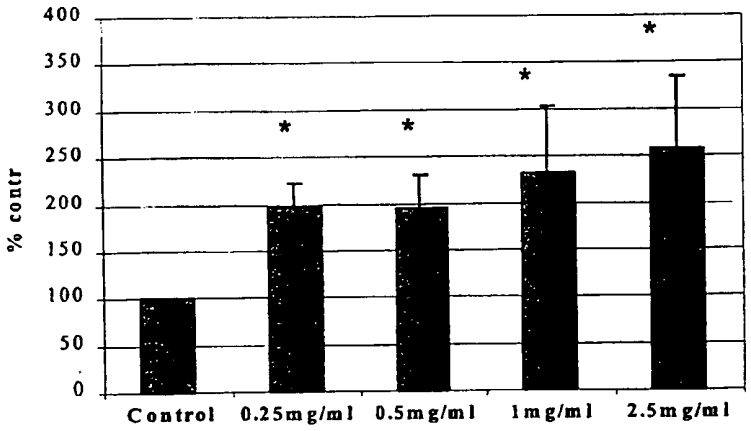


Figure 2

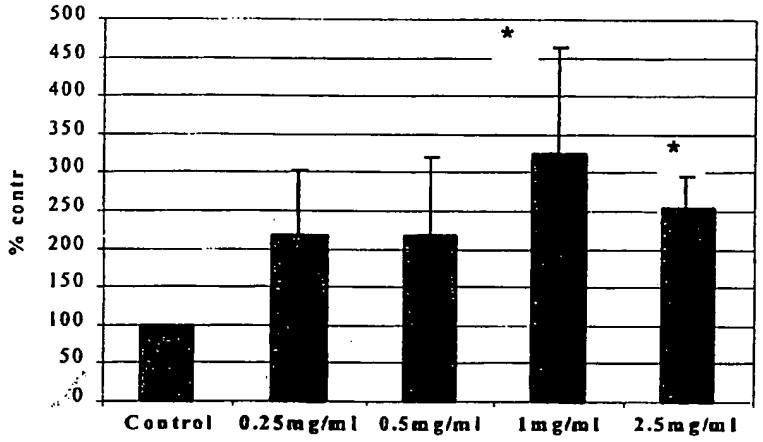


Figure 3

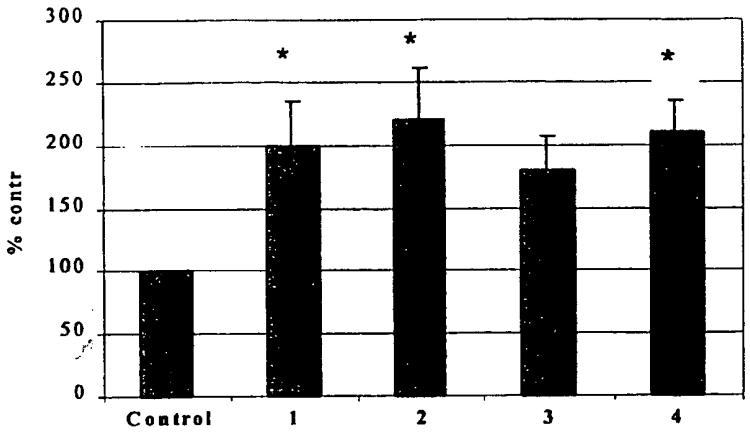
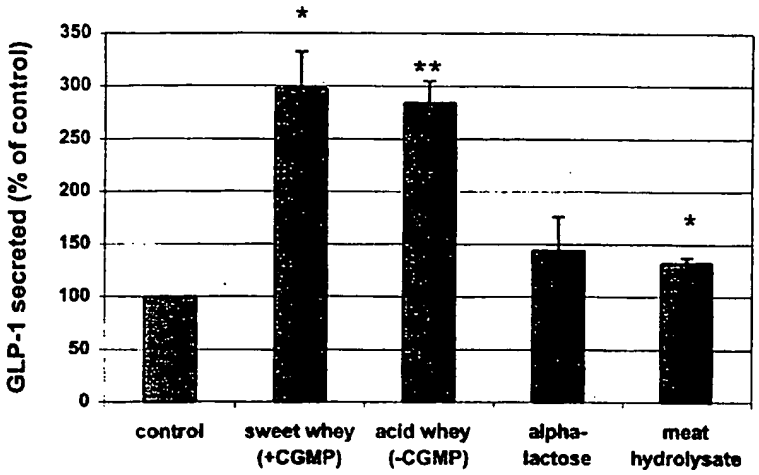


Figure 4



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(54) Title: USE OF A MILK PROTEIN HYDROLYSATE IN THE TREATMENT OF DIABETES

(57) Abstract: Use of a milk protein hydrolysate which is preferably a whey protein hydrolysate or caseinoglycomacropeptide (CGMP) in a bioavailable form in the manufacture of a composition for the treatment or prevention of diabetes or syndrome X and a method of treatment or prevention of diabetes or syndrome X are described. The present invention also relates to a method for assessing proglucagon gene expression and GLP-1 release by a cell line derived from an adenocarcinoma of human caecum.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/01 C12Q1/68 G01N33/50 A61P3/10

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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